

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE



In re application of

Eiko MASATSUJI, et al.

Appln. No.: 09/492,763

Group Art Unit: 1614

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For: DERMAL AGENT

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents  
Washington, D.C. 20231

Sir:

I, Toshi Tsuzuki, hereby declare and state:

THAT I am a citizen of Japan;

THAT I have received the degree of Bachelor of Science in Chemistry in 1982 from  
Tokyo Institute of Technology in Tokyo, Japan;

THAT I have been employed by Showa Denko K.K. since 1996, where I hold a position  
as Senior Researcher in the Central Research Laboratory. Prior to my employment with Showa  
Denko, K.K., I served as a research fellow at the Rosenstiel School of Marine and Atmospheric  
Science at the University of Miami in Miami, Florida from 1994-1996; Researcher with Showa  
Denko, K.K. from 1990 -1994 in the Central Research Laboratory, and as a researcher with Life  
Science Research Laboratory of Showa Denko K.K.;

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THAT I work mainly in the fields of Biochemistry , Microbiology and Bioengineering and I specialize in the field of biochemistry of ascorbic acid derivatives, tocopherol and its derivatives and steroids.

THAT I am a co-inventor of the above-identified patent application;

I submit this Declaration in support of the patentability of our invention;

The following experiments to compare the inhibitory effect of ascorbic acid-2-phosphate zinc salt as claimed when compared to other ascorbic acid phosphate salts were conducted by me or under my direction and control.

## **EXPERIMENTATION**

### **Experiment 1**

A test using a three-dimensional reconstruction culture skin cell model was performed as follows to compare the skin primary irritation of zinc ascorbate (ASZ), a mixture of sodium ascorbate and zinc salt (APNa + Z), zinc ascorbic acid-2-phosphate (APZ), and a mixture of sodium ascorbic acid-2-phosphate and zinc salt (APNa + Z).

Each test substance was added to and dissolved in a 20 mM phosphate buffer (pH: 7.0) containing 150 mM sodium chloride, to a concentration shown in Table 1 below and the obtained solutions were used as test stock solutions. Each of these stock solutions was diluted stepwise with a common ratio of 3.16 to prepare test solutions having a concentration after the 1,000-fold or less dilution of the test stock solution. The ascorbic acid moiety and zinc contained in these test solutions had the same molar concentrations.

(Table 1) Test Solutions

Test Substance	Test Stock Solution, Concentration Added (w/w%)						
	As	AsZ	AP	APZ	As+Z	AP+Z	Z
Sodium ascorbate	17	-	-	-	17	-	-
Zinc ascorbate	-	21	-	-	-	-	-
Sodium ascorbic acid-2-phosphate	-	-	28	-	-	28	-
Zinc ascorbic acid-2-phosphate	-	-	-	30	-	-	-
Zinc chloride	-	-	-	-	17	17	17

The test solution and the above-described buffer solution each in 0.1 ml were superposed one on another on a three-dimensional culture skin model (produced by Gunze Ltd., diameter: 9 mm) and the skin model was cultured in a carbon dioxide gas incubator at 37°C for 24 hours. After the culturing, the test solution was removed and the skin model was washed with a phosphate buffer. This washed skin model was then cultured on a culture solution in a carbon dioxide gas incubator at 37°C for 16 hours. Thereafter, the skin model was transferred to a culture solution containing 0.5 mg/ml of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) and further cultured in a carbon dioxide gas incubator at 37°C for 3 hours. The precipitated formazan was extracted with isopropanol containing 0.04N hydrochloric acid and subjected to colorimetry using a wavelength of 570 nm.

From the measured values, the survival rate of the cultured cells when each solution was added was calculated and plotted with respect to the concentration. By fitting this to sigmoid curve, the concentration of each test solution for 50% survival was determined. The results are shown in Table 2 below.

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(Table 2) Concentration for 50% Survival	
Test Solution	Concentration for 50% Survival (dilution Magnification from test stock solution)
As	4.5
AsZ	313.2
APNa	3.3
APZ	54.5
As+Z	348.5
APNa + Z	49.9
Z	298.3

As apparent from the results in this test, zinc ascorbic acid-2-phosphate (APZ) and a mixture of sodium ascorbic acid-2-phosphate and zinc salt (APNa+Z) have slight skin irritation as compared with zinc salt alone (Z), zinc ascorbate (AsZ) and a mixture of sodium ascorbate and zinc salt (As+Z).

#### (Experiment 2)

Under the same test conditions as in Example 4 of the present application, sodium ascorbic acid-2-phosphate (APNa), potassium ascorbic acid-2-phosphate (APK) and aluminum ascorbic acid-2-phosphate (APAl) were examined on the antibacterial activity against *Propionibacterium acnes* JCM6425.

As a result, the minimum inhibitory concentration (MIC) against this strain was 10 mg/ml or more in all of these compounds.

By this experiment, the antibacterial activity of zinc ascorbic acid-2-phosphate (APZ) against *Propionibacterium* was verified to be greatly high as compared with other ascorbic acid-2-phosphate salts.

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(Experiment 3)

Under the same test conditions as in Example 5 of the present application, sodium ascorbic acid-2-phosphate (APNa), potassium ascorbic acid-2-phosphate (APK) and aluminum ascorbic acid-2-phosphate (APAl) were examined on the antibacterial activity against *Staphylococcus aureus* 1F012732.

As a result, the minimum inhibitory concentration (MIC) against this strain was 10 mg/ml or more in all of these compounds.

By this experiment, the antibacterial activity of zinc ascorbic acid-2-phosphate (APZ) against *Staphylococcus* was verified to be greatly high as compared with other ascorbic acid-2-phosphate salts.

(Experiment 4)

Under the same test conditions as in Example 6 of the present application, sodium ascorbic acid-2-phosphate (APNa), potassium ascorbic acid-2-phosphate (APK) and aluminum ascorbic acid-2-phosphate (APAl) each in the form of a 10 mg/ml solution were examined on the inhibitory activity against lipase derived from *Pseudomonas*.

As a result, inhibitory activity against this enzyme was not recognized in these compounds.

By this experiment, the inhibitory activity of zinc ascorbic acid-2-phosphate (APZ) against lipase derived from *Pseudomonas* was verified to be greatly high as compared with other ascorbic acid-2-phosphate salts.

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(Experiment 5)

Under the same test conditions as in Example 7 of the present application, sodium ascorbic acid-2-phosphate (APNa), potassium ascorbic acid-2-phosphate (APK) and aluminum ascorbic acid-2-phosphate (APAl) each in the form of a 10 mg/ml solution were examined on the inhibitory activity against lipase derived from *Propionibacterium*.

As a result, inhibitory activity against this enzyme was not recognized in these compounds.

By this experiment, the inhibitory activity of zinc ascorbic acid-2-phosphate (APZ) against lipase derived from *Propionibacterium* was verified to be greatly high as compared with other ascorbic acid-2-phosphate salts.

(Experiment 6)

Under the same test conditions as in Example 8 of the present application, sodium ascorbic acid-2-phosphate (APNa), potassium ascorbic acid-2-phosphate (APK) and aluminum ascorbic acid-2-phosphate (APAl) each in the form of a 10 mg/ml solution were examined on the inhibitory activity against hyaluronidase derived from *Propionibacterium*.

As a result, inhibitory activity against this enzyme was APNa: 19%, APK: 31%, and APAl: 39%.

By this experiment, the inhibitory activity of zinc ascorbic acid-2-phosphate (APZ) against hyaluronidase derived from *Propionibacterium* was verified to be greatly high as compared with other ascorbic acid-2-phosphate salts.

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I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: \_\_\_\_\_

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Toshi Tsuzuki